



Characterization of plasma membrane-associated proteins from *Aedes albopictus* mosquito (C6/36) cells that mediate West Nile virus binding and infection

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Abstract

This study isolated and characterized the West Nile virus (WNV) putative receptor molecule(s) from *Aedes albopictus* mosquito (C6/36) cells. The binding of WNV to C6/36 cells was saturated with 5000 particles per cell. The entry of WNV into C6/36 cells was strongly inhibited when pretreated with proteinase K and to a lesser extent with sodium periodate. However, pretreatment of C6/36 cells with phospholipases, glycosidases, heparinases and neuramidase had no effect on virus entry. By using virus overlay protein blot assay, WNV was observed to bind to the 140-kDa, 95-kDa, 70-kDa and 55-kDa plasma membrane-associated molecules isolated from C6/36 cells. Murine antibodies generated against the 95-kDa and 70-kDa membrane proteins effectively blocked WNV, Japanese encephalitis virus (JEV) and Dengue virus (DV) serotype 2 infection in C6/36 cells. In addition, the binding of the recombinant-WNV envelope domain III protein to C6/36 cells can be inhibited by the anti-95-kDa and anti-70-kDa membrane protein antibodies. These data strongly supported the possibility that the 95-kDa and 70-kDa plasma membrane-associated proteins are part of a receptor complex for mosquito-borne flaviviruses (WNV, JEV and DV) on mosquito cells.

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Keywords: Flavivirus; Virus entry; Receptor; Mosquito cells; Virus binding

Introduction

West Nile virus is a small enveloped virus and a member of the Japanese encephalitis virus serocomplex of the genus *Flavivirus* in the family *Flaviviridae*. West Nile virus is the causative agent of West Nile fever. A spectrum of associated complications (meningo-encephalitis and acute flaccid paralysis) has caused many fatalities in immuno-compromised individual (Nedry and Mahon, 2003). The first known human case of WNV infection in the Western Hemisphere (New World) was reported in August 1999 (CDC, 1999). Since 1999, WNV has spread across the eastern and southern states and into central United States (O'Leary et al., 2004). It was speculated that the movement of WNV to the Western hemisphere was caused by

migratory birds that acted as introductory hosts and perhaps by infecting ornithophilic mosquitoes (Rappole et al., 2000). The WNV is transmitted to vertebrate hosts mainly from the inoculation of virus harboring mosquitoes of the *Culex*, *Aedes*, *Anopheles*, *Minomyia* and *Mansonia* species in Africa, Asia and the United States (Burke and Monath, 2001; Ilkal et al., 1997).

Mature WNV consists of three structural proteins: the large envelope glycoprotein (E), the nucleocapsid protein (C) and the membrane protein (M). Within the virion is a single-stranded positive sense, infectious RNA genome (Brinton, 2002). Crystallography data on the ectodomain of the E protein of tick borne encephalitis virus (Rey et al., 1995) revealed that the E protein forms homodimers arranged in a head-to-tail orientation and lies relatively flat on the virion surface. Each monomer of the E protein is folded into three distinct domains: a central domain designated as domain I, an elongated dimerization region

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designated as domain II and domain III having Ig-like modules (Rey et al., 1995). Domain II of the E protein is responsible for low pH-dependent fusion of the virus E protein to the late endosomal membrane during uncoating (Rey et al., 1995; Kuhn et al., 2002; Chu and Ng, 2004a). Domain III of the E protein is postulated to function as the receptor binding domain of the virus.

Chu et al. (2005) has recently shown the antagonist effect of soluble recombinant WNV E domain III protein on binding and entry of WNV into mammalian and mosquito cells. In addition, monoclonal and polyclonal antibodies generated against domain III of flavivirus E protein are highly effective in neutralizing the viruses (Volk et al., 2004; Beasley and Barrett, 2002; Crill and Roehrig, 2001; Chu et al., 2005). Mutagenesis of flavivirus domain III of the E protein also alters the entry process and virulence of viruses (Vlaycheva and Chambers, 2002; Lee and Lobigs, 2000).

A number of putative cellular receptors (glycosaminoglycans, DC-SIGN, laminin receptor, BiP, $\alpha V\beta 3$ integrin, Hsp70 and Hsp90) have been identified for mosquito-borne flaviviruses in different mammalian cell types (Chen et al., 1997; Navarro-Sanchez et al., 2003; Tassaneetrithep et al., 2003; Jindadamrongwech et al., 2004; Thepparit and Smith, 2004; Chu and Ng, 2004b; Reyes-Del Valle et al., 2005) but none has been identified in mosquito cells. Little is currently known about the initial interaction of mosquito-borne flaviviruses including WNV with mosquito cells. Thus, this study attempts to isolate and characterize putative plasma membrane-associated molecule(s) from mosquito cells (C6/36) that bind and mediate infectious entry of WNV.

Results

Binding of WNV to C6/36 cells

To determine the involvement of receptor–ligand binding of WNV onto C6/36 cells, a virus–cell binding assay was first carried out. A constant number of C6/36 cell (1×10^3 cells) was incubated with increasing amounts of ^{35}S -methionine-labeled WNV for a period of 1 h. Unbound or excess virus was removed by washing extensively with PBS. The amount of virus bound to cells was quantified in triplicate by scintillation counting. The data showed that WNV binding to C6/36 cells can be saturated at levels of approximately 5000 virus particles per cell within 1 h of virus inoculation (Fig. 1). The binding of WNV to C6/36 cells was both dosage-dependent and occurred in a saturable fashion, suggesting the importance of the receptor-dependent binding of WNV to C6/36 cells.

Isolation of putative WNV binding receptor molecule(s) from the plasma membrane of C6/36 cells

Virus overlay protein blot assay (VOPBA) was first used to identify the putative receptor molecule(s) that bind WNV

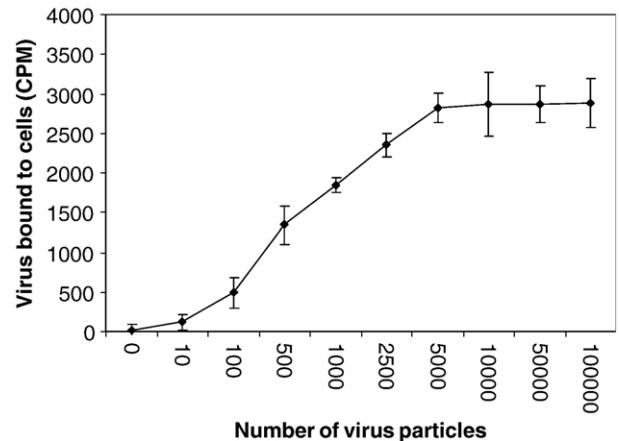


Fig. 1. Specific binding of ^{35}S -methionine-labeled WNV to the surface of C6/36 cells. The saturation of WNV binding to C6/36 cells can be achieved at approximately 5000 virus particles per cell. The radioactive counts from triplicates are averaged.

from the prepared plasma membrane proteins of C6/36 cells. Virus overlay protein blot assay was used successfully to identify and characterize receptor molecules for a number of viruses in different cell types (Choi et al., 1990; Borrow and Oldstone, 1992; Cao et al., 1998; Bruett et al., 2000; Chu and Ng, 2003). Equal quantities of the plasma membrane proteins were separated by SDS-PAGE and transferred onto nitrocellulose membrane. The membrane were cut into strips and incubated with radiolabeled WNV. Binding of the radiolabeled virus on the membrane is detected by exposing the membrane strips to X-ray films.

Using VOPBA, WNV was observed to bind to a series of protein bands of different molecular masses (55 kDa, 70 kDa, 95 kDa and 140 kDa) in the plasma membrane preparations of C6/36 cells (Fig. 2a). The 95-kDa and 70-kDa protein bands exhibited much stronger virus binding as compared to the rest of the protein bands. To ensure that these bands were not contributed by the non-specific binding of residual radioisotopes in the supernatant, the supernatants of uninfected cells prepared accordingly to the WNV-infected cells were also incubated under the same conditions. No bands were observed on the membrane incubated with the supernatant of uninfected cells (Fig. 2b). The specificity of the radiolabeled virus binding to the series of bands was further confirmed by using a high-salt buffer wash (with 200 mM of NaCl). The WNV binding to the 140-kDa and 55-kDa bands was no longer detectable after the membrane strip was being washed with the high-salt buffer (Fig. 2c), hence indicating that WNV has lower affinity binding or non-specific binding to the 140-kDa and 55-kDa protein bands.

Biochemical characterization of putative West Nile virus receptor molecule(s) on C6/36 cells

Two-pronged approaches (cellular and VOPBA) were used to characterize the biochemical nature of these

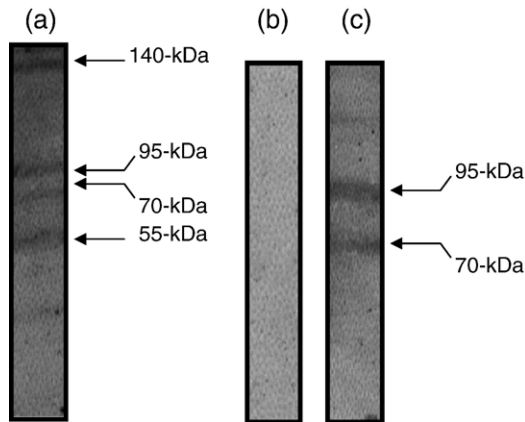


Fig. 2. West Nile virus binds to isolated plasma membrane-associated proteins from C6/36 cells using VOPBA. Plasma membrane-associated proteins from C6/36 cells were extracted under non-denaturing conditions, separated by SDS-PAGE and transferred to nitrocellulose membrane. ^{35}S -methionine-labeled WNV binding to the membrane protein bands was detected by exposing to X-ray film. (a) WNV binds to C6/36 plasma membrane-associated protein bands of molecular masses 140 kDa, 95 kDa, 70 kDa and 55 kDa. (b) No band is observed from proteins purified from supernatants of ^{35}S -methionine-labeled radio-labeled uninfected cells, hence indicating the specific binding of WNV to the plasma membrane proteins of C6/36 cells. (c) Highly specific binding of WNV to the 95-kDa and 70-kDa membrane protein bands is noted in the presence of high stringent washing conditions (200 mM of NaCl and 0.05% Tween 20). Binding of WNV to the 140-kDa and 55-kDa membrane protein bands is totally abolished under these stringent conditions. Molecular size markers are indicated on the right of each membrane blot.

putative WNV receptor molecules on C6/36 cells. The biochemical nature of the putative WNV receptor molecule(s) on the surface of intact mosquito (C6/36) cells was first characterized by pre-treating C6/36 cells with a panel of enzymes (phospholipases, proteases and glycosidases) or sodium periodate that would destroy the individual membrane components (e.g., lipids, proteins and carbohydrates). The concentrations of the enzymes and sodium periodate used in this study have been shown to effectively inhibit the entry of several viruses (Borrow and Oldstone, 1992; Ramos-Castaneda et al., 1997; Chu and Ng, 2003). The treated C6/36 cells were tested for cellular viability by Cytotox 96 non-radioactive cytotoxicity assay kit (Promega) and morphological observation under the microscope before proceeding for WNV infection. Minimal cytotoxicity was observed in the treated C6/36 cells. The ability of the treated cells to mediate WNV binding and subsequent infectious entry was determined by plaque assays. Results shown in Fig. 3 were expressed as the number of \log_{10} unit inhibition with respect to the untreated samples.

Treatment of C6/36 cells with the three phospholipases (A, C and D) did not substantially inhibit the infectious entry of WNV (Fig. 3a). Furthermore, pretreatment of C6/36 cells with glycosidases (α -mannosidase, endoglycosidase, α -fucosidase, *O*-glycosidase, neuraminidase), heparinases and lectins (wheat germ agglutinin, concanavalin A, phytohe-

magglutinin) did not significantly prevent the infectious entry of WNV into C6/36 cells (Figs. 3b and c, respectively). Despite the lack of inhibition of WNV entry after the treatment of C6/36 cells with a different range of glycosidases, sodium periodate (acts by oxidizing cell-surface carbohydrate residues while not affecting cell surface protein or lipid epitopes) was capable of reducing WNV entry into C6/36 cells in a dosage-dependent manner (Fig. 3b). In addition, pretreatment of C6/36 cells with both serine and thiol proteases strongly inhibit WNV entry in a dosage-dependent manner (Fig. 3d). Proteinase K (an endolytic protease that cleaves peptide bonds) strongly inhibits WNV infection with approximately a 4- \log_{10} reduction. Based on the results obtained above, it suggests that the WNV receptor molecule(s) on the surface of C6/36 cells are predominantly proteinaceous in nature and with possibility of some degree of glycosylation (sensitive to sodium periodate).

To affirm the above observations, the biochemical nature of the putative WNV binding receptor molecule(s) was also reassessed via VOPBA. Fig. 4a shows the binding of WNV to the 140-kDa, 95-kDa, 70-kDa and 55-kDa membrane protein bands in the absence of biochemical treatments. Proteinase K treatment of the separated C6/36 plasma membrane-associated proteins reduced WNV binding at the concentration of 0.01 $\mu\text{g}/\text{ml}$ (Fig. 4b) and totally abolished WNV binding to all the protein bands at the concentration of 0.1 $\mu\text{g}/\text{ml}$ (Fig. 4c), whereas treatment of the membrane proteins with phospholipases did not affect virus binding to all the protein bands (Fig. 4d). However, the binding of WNV to the 95-kDa, 70-kDa and 55-kDa plasma membrane-associated proteins was drastically reduced upon treatment with 10 mM sodium periodate (Fig. 4e) but not for the 140-kDa protein band.

Inhibition of WNV entry into C6/36 cells with murine polyclonal antibodies against the putative WNV receptor molecules

The 140-kDa, 95-kDa, 70-kDa and 55-kDa plasma membrane proteins isolated from C6/36 cells were used to generate polyclonal antibodies in mice. The crude murine sera were purified using columns to obtain IgG antibodies for the subsequent experiments. The specificity of the murine polyclonal antibodies generated against these isolated plasma membrane proteins was checked by Western blot assay. Antibodies against the different molecular mass proteins were able to detect their respective membrane proteins specifically (Fig. 5, Lanes 1, 3, 5 and 7). No bands were detected with the separated membrane proteins when incubated with pre-immune sera (Fig. 5, Lanes 2, 4, 6 and 8).

To affirm the role of these putative membrane proteins in mediating the entry of WNV into C6/36 cells, a series of WNV entry blockage studies were performed with the

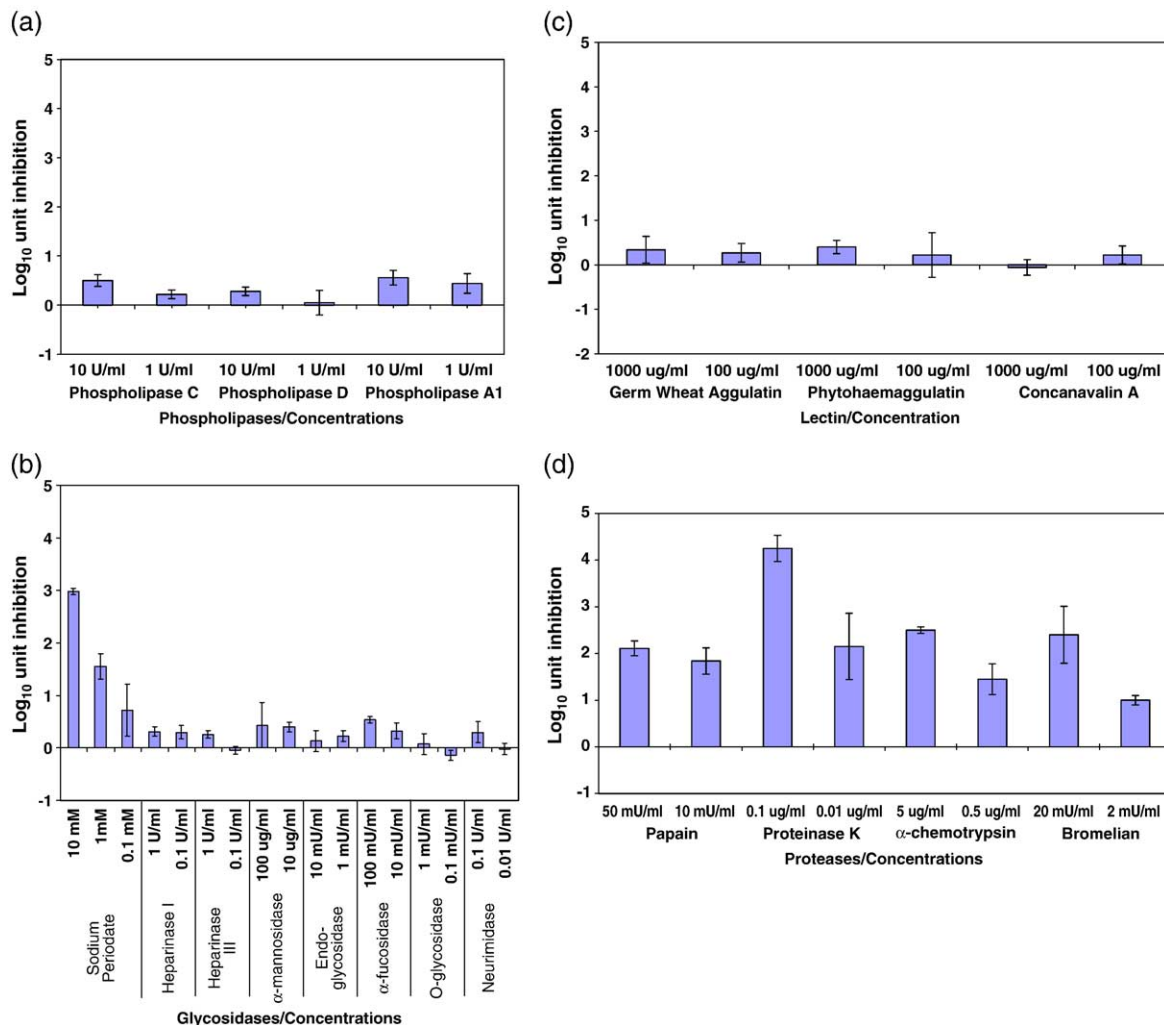


Fig. 3. Biochemical characterization of WNV-binding molecules present on the surface of intact C6/36 cells. (a) Pretreatment of C6/36 cells with phospholipases does not significantly reduce the infectious entry of WNV. In addition, WNV infection is resistant to the effect of (b) glycosidases, heparinases and (c) lectins. However, C6/36 cells pretreated with sodium periodate (b) are capable of inhibiting the infectious entry of WNV in a dosage-dependent manner. (d) Pretreatment of C6/36 cells with a panel of proteases exhibited significant inhibition of WNV infectious entry in a dosage-dependent manner. Proteinase K has the most potent inhibitory effect on WNV infection, resulting in 4-log unit reduction of virus yield at the concentration of 0.1 μ g/ml.

murine polyclonal antibodies generated against the 140-kDa, 95-kDa, 70-kDa and 55-kDa plasma membrane-associated proteins. The specific membrane protein antibodies were first incubated with C6/36 cells before virus infection (WNV, JEV, DV and Poliovirus). Fig. 6a shows that murine polyclonal antibodies against 70-kDa membrane proteins exhibited the strongest inhibitory effect on the binding and entry of WNV into C6/36 cells. The antibodies against the 95-kDa membrane protein was also capable of reducing WNV entry into C6/36 cells while antibodies against the 140-kDa and 55-kDa membrane proteins had minimal inhibitory effect on WNV binding and subsequent entry into C6/36 cells.

The localization of WNV with the 95-kDa or 70-kDa membrane proteins was assessed by immunofluorescence assays. WNV was first incubated with C6/36 cells for 30 min at 4 °C and processed for double indirect immunostain-

ing with antibodies against the membrane proteins (95 kDa and 70 kDa) and WNV. Figs. 6b and c show strong co-localization signal of WNV with the 95-kDa and 70-kDa membrane proteins, respectively. West Nile virus was observed in close association with the 95-kDa and 70-kDa membrane proteins (arrows) which exhibited typical localization pattern of plasma membrane-associated proteins.

Interestingly, the entry of both JEV and DV (serotype 2) was also significantly reduced by the anti-70-kDa and anti-95-kDa membrane protein antibodies in a similar trend as with WNV (Figs. 6d and e, respectively). In contrast, the entry of poliovirus (non-related virus) was not affected by the antibodies' blockage against all the membrane proteins (Fig. 6f). Therefore, these results suggested that the 70-kDa and 95-kDa plasma membrane-associated proteins played a role as common receptor molecules for mediating the entry of WNV, JEV and DV into C6/36 cells.

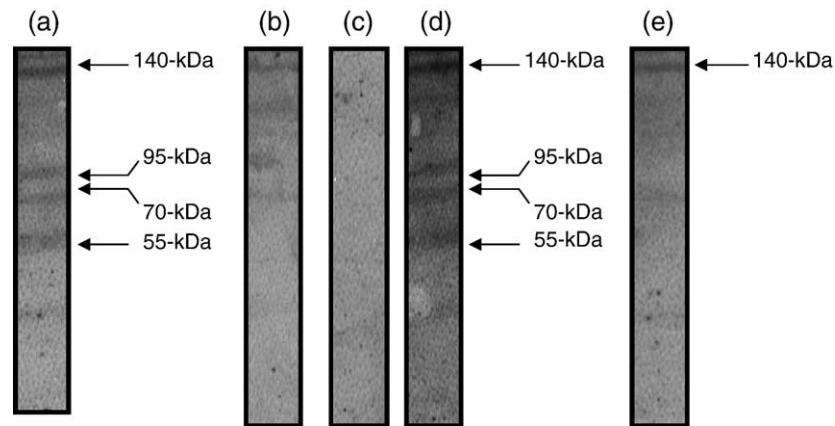


Fig. 4. Biochemical characterization of WNV-binding plasma membrane-associated protein on VOPBA. (a) WNV binds to the C6/36 membrane protein of 140 kDa, 95 kDa, 70 kDa and 55 kDa in the absence of enzymes or biochemical treatments. (b) Proteinase K (0.01 µg/ml) treatment of the membrane proteins on nitrocellulose membrane reduces WNV binding to all the membrane protein bands isolated from C6/36 cells. (c) The binding of WNV to the membrane protein was totally abolished after treatment with proteinase K at the concentration of 0.1 µg/ml. No bands are observed. (d) WNV binding to the 140-kDa, 95-kDa, 70-kDa and 55-kDa membrane proteins is not affected after phospholipase A treatment (10 U/ml). (e) Pretreatment of the C6/36 plasma membrane proteins with 10 mM of sodium peroxide shows inhibition of WNV binding to the 95-kDa, 70-kDa and 55-kDa membrane protein bands but has no effect on the 140-kDa protein band. Molecular size markers are indicated on the right of each membrane blot.

Anti-95-kDa and anti-70-kDa membrane protein polyclonal antibodies blocked recombinant WNV E domain III protein binding to C6/36 cells

Recent studies have identified that the recombinant WNV E domain III of the flavivirus envelope protein is responsible for binding to cellular receptors (Mukhopadhyay et al., 2003; Volk et al., 2004; Chu et al., 2005). The ability of antibodies against the putative receptor molecule(s) blocking the binding of WNV E domain III protein was assessed. West Nile virus E domain III protein was expressed, purified and labeled with FITC as described in Materials and methods. C6/36 cells were pre-incubated with antibodies against the 140-kDa, 95-kDa, 70-kDa or 55-kDa membrane proteins followed by the FITC-labeled WNV E domain III protein. Both antibodies against the 95-kDa and 70-kDa membrane proteins substantially blocked the binding of DIII protein to

C6/36 cells in a dosage-dependent manner (Fig. 7). However, minimal blockage of FITC-labeled WNV E domain III protein to the C6/36 cells was observed by the anti-140-kDa and anti-55-kDa membrane protein antibodies.

Together, these results have provided strong evidence that the 95-kDa and 70-kDa membrane proteins are possible cellular receptor or part of the receptor complex for WNV and, possibly, JEV and DV (serotype 2) into C6/36 cells.

Discussion

The infectious entry of virus into host cells is mediated by the initial interaction of the virus attachment protein (VAP) and the cellular receptor molecules. The interaction of VAP and its cellular counterpart is known to contribute to host range, tissue tropism and viral pathogenesis. Extensive

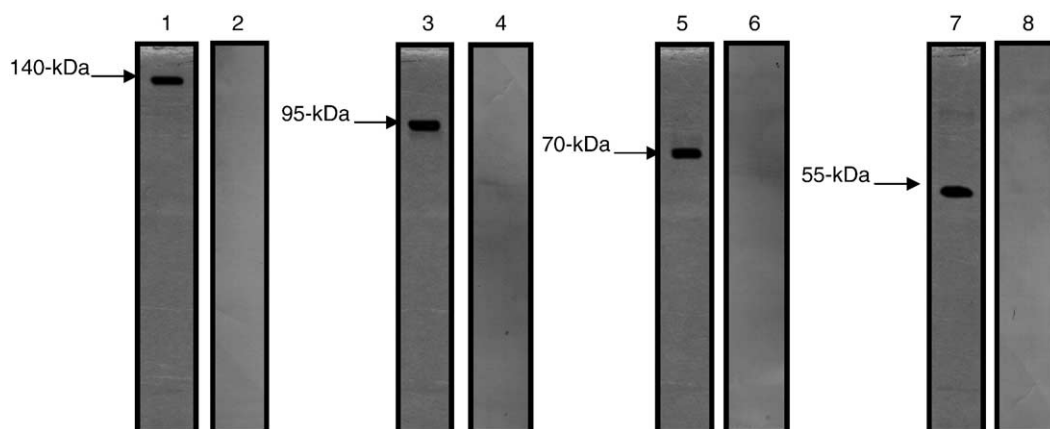
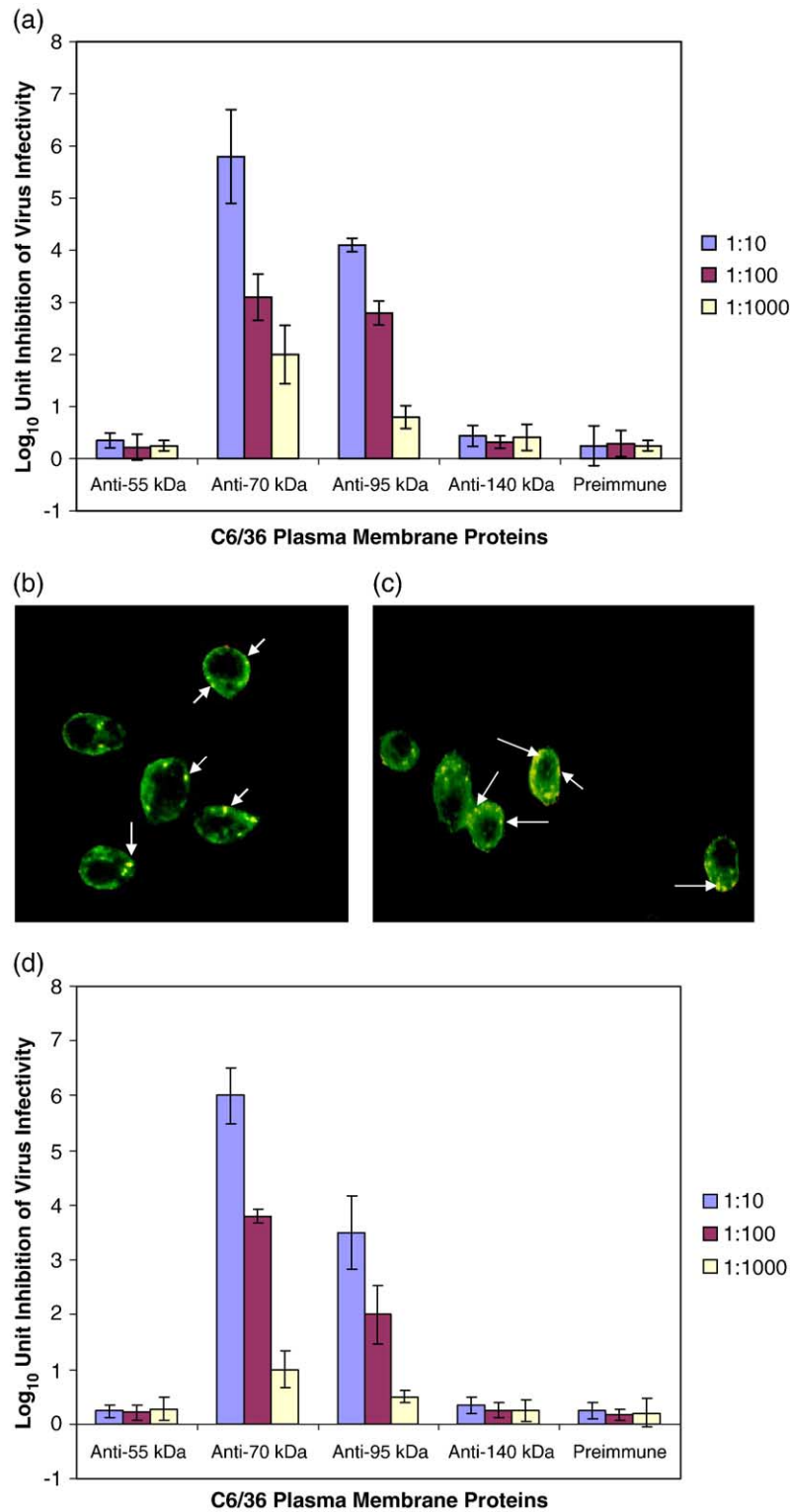


Fig. 5. Immuno-detection of C6/36 membrane proteins with murine polyclonal antibodies produced from plasma membrane-associated protein extract of C6/36 cells. Detection of C6/36 membrane proteins with pre-immune sera (Lanes 2, 4, 6 and 8) and the anti-140-kDa (Lane 1), anti-95-kDa (Lane 3), anti-70-kDa (Lane 5) and anti-55-kDa (Lane 7) protein polyclonal antibodies was performed. The anti-140-kDa, 95-kDa, 70-kDa and 55-kDa murine polyclonal antibodies generated are highly specific for its respective membrane proteins from C6/36 cells. Molecular size markers are indicated on the left of each membrane blot.

studies have been carried out to determine the infectious entry process of mosquito-borne flaviviruses into different mammalian cell types (Anderson, 2003). So far, not much is known about the initial interaction of mosquito-borne flaviviruses with mosquito cells.

To our knowledge, this is the first study that characterized the putative cellular counterparts from mosquito cells

that mediate WNV binding and entry. Studies have shown that WNV can be isolated from *Culex*, *Aedes*, *Anopheles*, *Minomyia* and *Mansonia* mosquitoes (Burke and Monath, 2001; Ilkal et al., 1997). Hence, this study utilized the *Aedes albopictus* cell line (C6/36) that has been shown to be highly susceptible to WNV infection (Turell et al., 2001), and WNV has also been isolated from field strains of *A.*



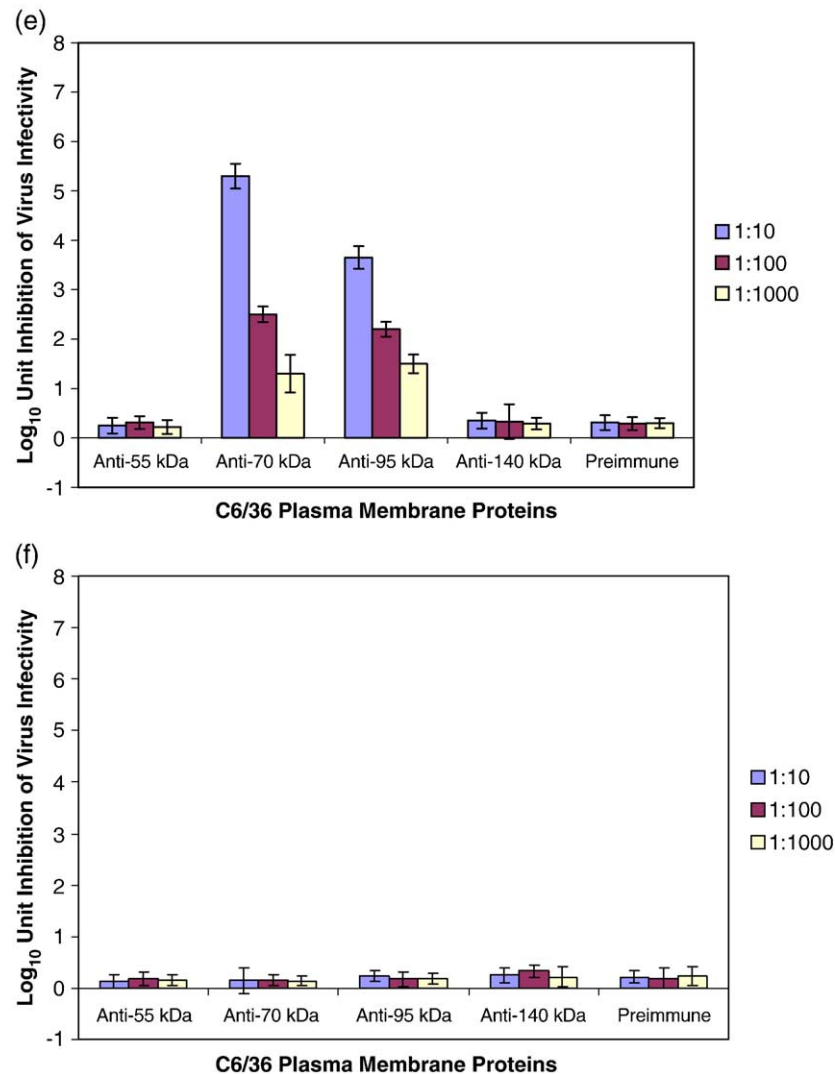


Fig. 6. West Nile virus interacts with the 95-kDa and 70-kDa plasma membrane-associated proteins of C6/36 cells. Antibody blockage assay of WNV infectious entry was carried out using antibodies against the 140-kDa, 95-kDa, 70-kDa and 55-kDa membrane proteins. (a) A dosage-dependent inhibition of WNV infection with the anti-95-kDa and anti-70-kDa membrane protein antibodies is noted but not with antibodies generated against the 140-kDa and 55-kDa membrane proteins. The immuno-fluorescence detection of WNV binding to the (b) 95-kDa (arrows) and (c) 70-kDa (arrows) plasma membrane-associated proteins is shown. WNV is stained with Texas Red-conjugated monoclonal antibodies and the plasma membrane 95-kDa and 70-kDa receptor proteins are stained with FITC-conjugated respective polyclonal antibodies. The co-localization of WNV particles with the 95-kDa or 70-kDa membrane proteins (dots) can be clearly observed. Similarly, the murine polyclonal antibodies against the 95-kDa or the 70-kDa membrane proteins are capable of reducing (d) JEV and (e) DV infection in C6/36 cells. (f) In contrast, poliovirus infection of C6/36 cells is not affected in the presence of antibodies against the panel of WNV-binding membrane proteins. Triplicate results are expressed as log₁₀ unit reduction of WNV infection at a given antibody concentration. Preimmune sera are included to ensure that the inhibitory effect on WNV infection is not due to non-specific blockage by the murine antibodies.

albopictus mosquitoes. The ability of flavivirus to infect a cell line derived from a particular arthropod vector can reflect the natural vector-virus relationships (Mussgay et al., 1975; Lawrie et al., 2004), thus providing information about virus transmission and viral persistence in the natural environment. In this study, WNV was shown to bind specifically to C6/36 cells in a dose-dependent and saturable manner with approximately 5000 virus particles per cell (Fig. 1). These results displayed the characteristics of typical ligand-receptor interactions existing, hence suggesting the presence of a specific interaction between WNV and receptor molecule(s) on C6/36 cells.

Glycoaminoglycans have been implicated in mediating the attachment and entry of mosquito-borne flaviviruses into mammalian cells (Chen et al., 1997; Lee and Lobigs, 2000). It is postulated that the initial binding of flaviviruses to the cellular surface is mediated by heparan sulfate and subsequent entry occurring through a high-affinity receptor (Putnak et al., 1997). However, the involvement of heparan sulfate in mediating the attachment of flaviviruses to cell surface is not universal, as it could be dependent on the member of the flavivirus family, the virus strains, the passage level of the virus and the cell types (Bielefeldt-Ohmann et al., 2001;

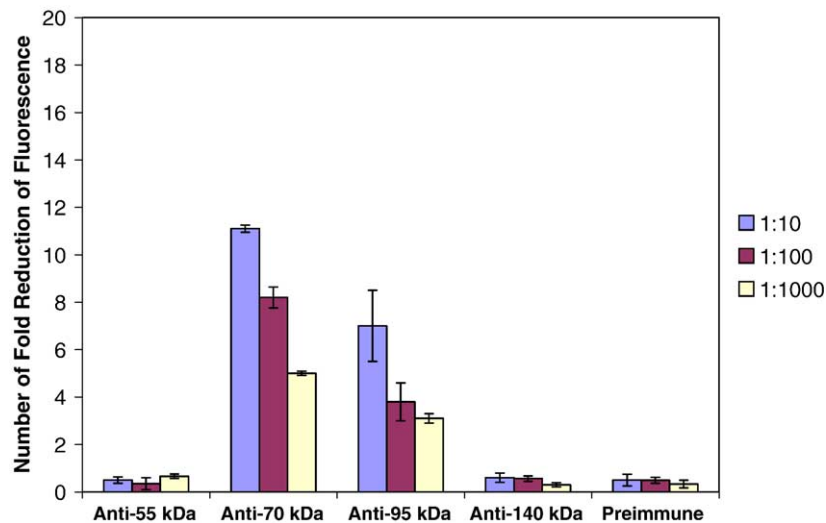


Fig. 7. Recombinant WNV E DIII protein binds to the 95-kDa and 70-kDa putative receptor molecules on C6/36 cells. A dosage-dependent inhibition of FITC-labeled WNV E DIII protein can be observed in C6/36 cells with pre-incubation of cells with antibodies against the 95-kDa or 70-kDa putative receptor molecules. In contrast, antibodies specific for the 140-kDa and 55-kDa and the pre-immune sera have minimal effect on WNV E DIII protein binding to C6/36 cells.

Kroschewski et al., 2003; Lin et al., 2002; Chu and Ng, 2003).

A recent study by Hung et al. (2004) demonstrated that the binding of dengue virus envelope domain III (putative receptor binding domain) to C6/36 cells is independent of heparan sulfate. In this study, the attachment of WNV to heparan sulfate in C6/36 cells was assessed by pretreatment of the cells with heparinases. The infectious entry of WNV into C6/36 cells was not affected despite the pretreatment of C6/36 cells with heparinases (Fig. 3). A previous study by Chu and Ng (2003) has also shown that the binding of WNV to Vero cells does not require heparan sulfate. Therefore, it seems that heparan sulfate is not required for WNV entry in both mosquito and vertebrate cells.

Sialic acids present on the surface of cells are known to function as attachment factor and cellular receptor mediating the entry of a number of viruses that include influenza viruses (Suzuki, 2001), rotavirus (Arias et al., 2002), bovine parvovirus (Johnson et al., 2004) and equine rhinitis A virus (Stevenson et al., 2004). The infection of C6/36 cells with WNV was resistant to neuraminidase pre-treatment, suggesting that sialic acid is not essential for virus binding and subsequent virus entry into cells (Fig. 3). Salas-Benito and del Angel (1997) has also reported that treatment of C6/36 cells with neuraminidase does not affect the binding of dengue virus (serotype 4) to cells.

In addition to the utilization of glycoaminoglycans and sialic acid as cellular receptors for virus entry into cells, carbohydrate residues on cellular receptors have also been reported to be essential for virus binding to the receptors (Chu and Whittaker, 2004; Ferreira et al., 2004; Spiropoulou et al., 2002; Smith and Helenius, 2004). Our previous study has shown the importance of N-linked sugars with α -mannose residues on the cellular receptor of Vero and murine neuroblastoma cells for WNV binding

(Chu and Ng, 2003). However, pretreatment of intact C6/36 cells with a panel of glycosidases that include endoglycosidase H, *O*-glycosidase, α -mannosidase, α -fucosidase and competitive assays with lectins (concanavalin-A and phytohemagglutinin) did not exert any effect on inhibiting the infectious entry of WNV (Fig. 3). It seems consistent that carbohydrate residues on the surface of C6/36 cells are not necessary for the binding of WNV and dengue virus (serotype 4) (Salas-Benito and del Angel, 1997).

A number of putative WNV-binding plasma membrane-associated molecules (140 kDa, 95 kDa, 70 kDa and 55 kDa) from C6/36 cells were demonstrated by VOPBA in this study (Fig. 2a). The technique of VOPBA has been used successfully in the identification of virus receptors on cell surfaces (Choi et al., 1990; Borrow and Oldstone, 1992; Cao et al., 1998; Bruett et al., 2000; Chu and Ng, 2003). West Nile virus was observed to bind specifically to two protein bands (95 kDa and 70 kDa) from C6/36 cells under high stringency conditions of high-salt (200 mM) and -detergent (0.05% Tween 20) washing (Fig. 2b). Biochemical analysis of these WNV-binding membrane proteins from C6/36 cells with proteases and sodium periodate treatment (Fig. 3) strongly suggested the glycoproteineous nature of the putative receptor molecules.

The production of murine polyclonal antibodies against the 140-kDa, 95-kDa, 70-kDa and 55-kDa C6/36 cell plasma membrane-associated proteins showed that polyclonal antibodies against the 95-kDa and 70-kDa proteins were capable of inhibiting the infectious entry of WNV, JEV and DV (serotype 2) in a dosage-dependent manner. This result was consistent with a previous study by Chu et al. (2005) showing that both WNV and DV (serotype 2) may utilize the same putative receptor molecule on the surface of C6/36 cells for virus binding and subsequent entry. The

specificity of the 95-kDa and 70-kDa plasma membrane-associated proteins in mediating WNV binding and entry was further supported by immunofluorescence assay whereby the binding of WNV to the 95-kDa or 70-kDa membrane proteins was observed on the cell surface of C6/36 cells (Figs. 6b and c). Furthermore, minimal blocking effect on the infectious entry of the unrelated poliovirus (Fig. 6f) was achieved when C6/36 cells were pre-incubated with antibodies against the 95-kDa and 70-kDa membrane proteins.

However, antibodies against the 140-kDa and 55-kDa membrane proteins (Fig. 6) were not effective in blocking the infection of C6/36 cells by WNV, JEV and DV (serotype 2). It was interesting to observe that WNV could bind to the 140-kDa and 55-kDa membrane proteins in VOPBA but the antibodies specific for the 140-kDa and 55-kDa membrane proteins failed to prevent infectious entry of WNV into C6/36 cells. A possible explanation is that WNV binds to the 140-kDa and 55-kDa membrane proteins that may associate with the receptor complex that were extracted in the procedure for VOPBA. However, these membrane proteins may not serve as the functional receptor to mediate the entry of WNV into C6/36 cells, hence, antibodies generated against the 140-kDa and 55-kDa membrane proteins had no effect on blocking virus entry into C6/36 cells.

Domain III of the WNV E protein has been reported to serve as the receptor binding domain (Beasley and Barrett, 2002; Volk et al., 2004; Chu et al., 2005), and this study has also clearly illustrated that the WNV E domain III protein can bind to the 95-kDa and 70-kDa receptor molecules with high specificity. The preincubation of C6/36 cells with antibodies against the 95-kDa or the 70-kDa membrane proteins effectively prevented the binding of the WNV E domain III protein to C6/36 cells (Fig. 6).

Hence, these results showed the specific interactions of WNV, JEV and DV (serotype 2) with the 95-kDa and 70-kDa membrane proteins on C6/36 cells for the entry process. Currently, efforts are directed to determine the identity of the 95-kDa and 70-kDa membrane proteins via peptide sequencing. The identification of these putative receptor molecules would provide insight to their distributions in both susceptible and non-susceptible mosquito tissues, hence facilitating the development of vector control strategies.

Materials and methods

Maintenance of cell lines

C6/36 cells, a continuous mosquito cell line (kindly provided by Professor Akira Igarashi, Japan) derived from *A. albopictus* (Diptera: Culicidae) embryonic tissue, were grown in L-15 medium (GIBCO) containing 10% heat-inactivated fetal calf serum (FCS) at 28 °C. Vero cells (green

monkey kidney cells, America Type Culture Collection) were maintained at 37 °C in Medium 199 (M199; GIBCO) containing 10% FCS.

Virus growth, radiolabeling and purification

The flaviviruses used in this study were kindly provided by Professor Edwin Westaway, Australia. West Nile virus (Sarafend), Japanese encephalitis virus (JEV, Nakayama) and Dengue virus (New Guinea) were propagated in C6/36 cells throughout this study, and poliovirus was propagated in Vero cells. The quantification of the viruses was performed on Vero cells using plaque assay.

Confluent monolayers of C6/36 cells were infected with WNV at a multiplicity of infection (M.O.I.) of 10. For the preparation of radiolabeled WNV, at 10 h p.i., cells were starved in methionine-free medium for 2 h and the medium was then replaced by 0.5% FCS-L-15 media containing L-³⁵S-methionine [10 µCi/ml] and 2 µg/ml actinomycin D (Sigma, USA). At 48 h p.i., the infected cell culture supernatant (labeled or non-labeled virus) was harvested. The infected cell culture supernatant was centrifuged at 5000 rpm for 10 min to remove cell debris before the virus was concentrated and purified according to the process described below.

West Nile virus was first concentrated and partially purified by using a centrifugal filter device (Millipore, Bedford) at 2000 rpm for 2 h. The partially purified viruses were then applied onto a 5 ml 25% sucrose cushion for further purification. Sucrose gradient was centrifuged at 25,000 rpm for 2.5 h at 4 °C in a SW55 rotor. Finally, the purified virus pellet was resuspended in TNE buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.2). The resuspended virus was aliquoted, snapped frozen and stored at –80 °C. The specific infectivity and radioactivity of the radiolabeled virus preparation were approximately 3×10^8 PFU/ml and 9×10^3 cpm/ml, respectively.

Virus-cell binding assay

To determine the binding affinity of WNV to C6/36 cells, 1×10^3 C6/36 cells were incubated with increasing amounts of radiolabeled WNV at 4 °C for 1 h. Virus-cell interaction was carried out at 4 °C to avoid viral penetration. WNV is added to C6/36 cells in the range of 100, 1000, 2500, 5000, 10,000, 50,000 and 100,000 virus particles per cells. Excess unbound virus was removed from the cell surface by washing the cells with PBS for five times. The cells were then lysed with lysis buffer (10 mM PBS, pH 7.2, containing 1% Triton X-100 and 0.1% SDS) and the radioactivity of the cell lysate was determined in a scintillation counter. Specific binding of WNV to the C6/36 cells was determined by subtracting the bound CPM obtained after incubating with the radiolabeled WNV with the bound CPM due to non-specific binding (obtained from cells incubated with supernatant of uninfected cells purified

with the same procedure). The binding assay was carried out in triplicates.

Biochemical (protease, phospholipase, glycosidase and lectins) treatment of C6/36 cells

To assess the biochemical nature of the cellular receptor molecule(s) on the plasma membrane of C6/36 cells to which WNV binds, cell monolayers of approximately 8×10^5 cells were washed twice with PBS before enzyme treatment. Cell monolayers were incubated with the proteases, phospholipases, glycosidases, and lectins in PBS at pH 7.2 for 45 min at 25 °C. After treatment, cell monolayers were washed twice with PBS supplemented with 5% FCS to remove the enzymes. Cell monolayers were then incubated with WNV (M.O.I. = 10) at 37 °C for 1 h. Excess virus particles were inactivated with acid glycine buffer, pH 3.0, for 5 min and the cell monolayer was washed twice with PBS to remove inactivated viruses. C6/36 cells were then incubated at 28 °C for 48 h. At 48 h p.i., virus titers from the treated samples were determined by plaque assays.

The enzymes used in this study were as described by Chu and Ng (2003). In brief, the concentrations of the biochemical used were as follows—proteases: proteinase K (EC 3.4.21.64) from *Tritirachium album* (Sigma, USA), concentrations of 1 and 0.1 µg/ml; α-chymotrypsin (EC 3.4.21.1) from bovine pancreas (Sigma, USA), concentrations of 5 and 0.5 µg/ml; bromelain (EC 3.4.22.32) from pineapple stem (Sigma, USA), concentrations of 5, 2.5, and 0.2 mU/ml; papain (EC 3.4.22.2) from *Carica papaya* (Roche, USA), concentrations of 25 and 2.5 mU/ml; phospholipases: phospholipase A₂ (EC 3.1.1.4) from bovine pancreas (Sigma, USA), concentrations of 10 and 1 U/ml; phospholipase C (EC 3.1.1.4.3) from *Clostridium perfringens* (Sigma, USA), concentrations of 10 and 1 U/ml; phospholipase D (EC 3.1.4.4) from peanut (Sigma, USA), concentrations of 10 and 1 U/ml; glycosidases: endoglycosidase H (EC 3.2.1.96) from *Streptomyces plicatus* (Roche, USA), concentrations of 10 and 1 mU/ml; O-glycosidase (EC 3.2.1.97) from *Diplococcus pneumoniae* (Roche, USA), concentrations of 1 and 0.1 mU/ml; α-mannosidase (EC 3.2.1.24) from almonds (Sigma, USA), concentrations of 1000 and 100 µg/ml; α-fucosidase (EC 3.2.1.11) from almond meal (Sigma, USA), concentrations of 100 and 10 mU/ml; heparinase I (EC 4.2.2.7) and heparinase III (EC 4.2.2.2.8) from *Flavobacterium heparinum* (Sigma, USA), concentrations of 1 and 0.1 U/ml; lectins: concanavalin-A from Jack bean (Sigma, USA), wheat germ agglutinin from *Triticum vulgaris* (Sigma, USA), phytohemagglutinin from *Phaseolus* spp. (Wellcome Diagnostic, UK), concentrations of 1000 and 100 µg/ml. Sodium periodate was obtained from Sigma (USA) and the working concentrations are 10 and 1 mM. Cell viability after enzyme treatment was assessed by Cytotox 96 non-radioactive cytotoxicity assay kit (Promega, USA) and by

observation under phase-contrast microscope IX 81(Olympus, Japan).

Extraction and purification of C6/36 plasma membrane-associated proteins

The plasma membrane proteins were prepared as previously described by Martinez-Barragan and del Angel (2001). Monolayers of C6/36 cells were detached with PBS containing 5 mM EDTA for 10 min at room temperature. Afterwards, cells were resuspended in ice-cold buffer M (100 mM NaCl, 20 mM Tris [pH 8], 2 mM MgCl₂, 1 mM EDTA, and 1 mM β-mercaptoethanol) and lysed by 5 to 10 strokes in a tight fitting Dounce homogenizer. Nuclei and debris were removed by centrifugation at $500 \times g$ for 10 min at 4 °C in a Sorvall centrifuge. Membrane proteins were pelleted from the supernatant by centrifugation at 18,000 rpm for 30 min at 4 °C in a Beckman JA20 rotor and resuspended in buffer M without β-mercaptoethanol. The integrity of extracted membrane proteins was determined as described by Atkinson and Summers (1971). The concentration of the protein was determined by Bradford assay with bovine serum albumin [(BSA) (CSL, Australia)] as the standard. Approximately 750 µg of proteins was obtained. The membrane protein preparation was aliquoted and stored at –20 °C.

Virus overlay protein blot assay (VOPBA)

For the binding of WNV to putative molecules on the plasma membrane of C6/36 cells, VOPBA was carried out as essentially described by Martinez-Barragan and del Angel (2001). In brief, 80 µg of the plasma membrane proteins was first separated by 12% SDS-PAGE and electrophoretically transferred onto nitrocellulose membrane (Bio-Rad, USA). The nitrocellulose membrane was soaked overnight in a milk buffer (5% skim milk and 0.5% BSA—to block non-specific-binding sites and to allow renaturation of the separated proteins) before rinsing with PBS (three times) and incubated with the purified radiolabeled WNV (1×10^3 cpm/ml). Non-specific binding of the virus particles was reduced by washing with a high-salt buffer wash as previously described by Chu and Ng (2003). The presence of virus binding was detected by exposing the membranes to Kodak X-ray films. Finally, the films were developed, washed with distilled water and dried.

Enzymes, lectins and chemical treatments of C6/36 plasma membrane proteins on VOPBA

The plasma membrane proteins of C6/36 cells were first isolated and prepared for VOPBA as described in the previous paragraphs. Plasma membrane proteins were treated with proteinase K (0.01 µg/ml and 0.1 µg/ml), phospholipase A (10 U/ml), lectins sodium periodate (10 mM). The enzymatic effects of the enzymes were inacti-

vated with PBS containing 10% BSA. The membranes were then washed thrice with PBS before subjecting for WNV binding. Purified radiolabeled WNV (1×10^3 cpm/ml) was incubated with the membranes and the presence of virus binding was detected by exposing the membranes to Kodak X-ray films.

Generation of murine polyclonal antibodies against plasma membrane-associated proteins from C6/36 cells

The plasma membrane protein bands that bind WNV were excised from the 12% SDS-PAGE gels, homogenized, and eluted using electro-eluter (BIO-RAD). The eluted proteins were incubated with ImmunEasy mouse adjuvant (Qiagen, USA) at a concentration recommended by the manufacturer. The antigen–adjuvant mixture was used to immunize BALB/c mice (Animal Holding Unit, National University of Singapore) five times peritoneally at 14-day intervals. Mouse sera were collected 12 days after the last booster. Mouse sera were purified using Econo-Pac serum IgG purification kits (Bio-Rad, USA) and dialyzed overnight with PBS. The purified immunoglobulins were stored at -20°C . Sera were tested by Western blotting (Chu and Ng, 2003) and the protein bands were detected using chemiluminescence (Ho et al., 2005).

Immunofluorescence detection of putative WNV binding molecules on C6/36 cells

For immunofluorescence microscopy, cell monolayers (C6/36) were grown on coverslips in a 24-well tissue culture plate. The cells were fixed and processed for immunostaining as described in Chu and Ng (2003). The primary antibodies used were murine polyclonal anti-55-kDa, 70-kDa, 95-kDa and 140-kDa plasma membrane proteins (dilution of 1:100) and monoclonal anti-WNV (H546, Microbix Biosystems Inc, Canada) at a dilution of 1:1000. Fluorescein isothiocyanate (FITC) and Texas Red (TR)-conjugated secondary antibodies (Amersham Pharmacia Biotech) were used. The specimens were viewed under an inverted microscope (Olympus IX81) with oil immersion objective. Fluorescence images were captured by digital camera (Photometrics, USA) and MetaMorph software, version 6 (Universal Imaging Corporation, USA).

Antibodies blockage assay

Confluent monolayers of C6/36 cells were first washed thrice with PBS and incubated with pre-immune serum or the anti-55, 70-, 95-, 140-kDa polyclonal antibodies (1:10 to 1:1000 dilutions) for 1 h at 4°C . After incubation, cells were washed thrice with PBS and infected with WNV, JEV, DenV and poliovirus (M.O.I. = 10). At appropriate time p.i., supernatants from the virus-infected cells were processed for plaque assays. Poliovirus infection was used as a negative control. Similarly, the above procedure was

repeated with the replacement of WNV with purified recombinant WNV envelope domain III protein. WNV envelope domain III protein was expressed and purified as described in Chu et al. (2005). Recombinant WNV DIII protein was labeled with FITC using EZ-label FITC protein labeling kit from Pierce Biotechnology, USA, and was carried out according to the manufacturer's instructions. FITC-labeled WNV envelope DIII protein (750 $\mu\text{g/ml}$) was incubated with C6/36 cells after the addition of anti-55, 70-, 95-, 140-kDa polyclonal antibodies and pre-immune serum (at a dilution of 1:10, 1:100, and 1:1000). The binding of FITC-labeled WNV envelope DIII protein to C6/36 cells was quantified by measuring the amount of fluorescence emitted using a fluorescent plate reader. The excitation and emission wavelengths were set at 380 nm and 520 nm, respectively. The amount of fluorescence measured was expressed as the number of fold reduction with cells incubated with the anti-plasma membrane-associated proteins antibodies with respect to the cells incubated with pre-immune sera.

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